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Kidney360

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--Manuscript Draft--

Manuscript Number:	K360-2025-000113R2
Full Title:	IgA-Producing B Cells Exert Regulatory Function Through Granzyme B in Kidney Allograft Tolerance
Short Title:	IgA B Cells in Kidney Graft Tolerance
Article Type:	Original Research
Section/Category:	Transplantation
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	Hoa Le Mai (Writing – review & editing)
	Nataliya Yeremenko (Formal analysis; Writing – review & editing)
	Sophie Brouard (Conceptualization; Funding acquisition; Writing – review & editing)
Manuscript Classifications:	63: Chronic Allograft Rejection; 229: IGA; 235: Immunology; 255: Kidney; 354: Regulation; 404: Tolerance; 410: Transplant Outcomes; 412: Transplantation
Abstract:	Background: Tolerant kidney transplant recipients without immunosuppression have a high frequency of circulating granzyme B (GZMB)-expressing regulatory B cells (Breg). Since the precursors of these Bregs remain unknown and IgA-secreting B cells have been shown to have regulatory properties in different situations, we investigated the association between IgA- and GZMB-expressing B cells in a case-control study. Methods: 45 healthy volunteers and 31 kidney transplant recipients with either stable graft function under immunosuppression (n=10), antibody-mediated rejection (n=7) or tolerant patients (n=14) were included. Serum immunoglobulin concentrations as glycosylation were measured by ELISA, forms of IgA were examined by Western blot. Regulatory B cells were analyzed by single-cell RNA sequencing and multiparameter spectral flow cytometry. Their function was assessed in co-cultured with T cells. Results: Serum IgA concentration was elevated in tolerant patients compared to other transplanted patients, especially the non inflammatory IgA1 subclass, without changes in IgA size or glycosylation. Single cell transcriptomic analysis revealed higher IgA gene expression in GZMB expressing B cells and conversely higher GZMB gene expression in IgA expressing B cells. Phenotyping analysis by spectral multiparameter flow cytometry showed that IgA+ B cells were memory B cells and that IgA+ B cells from tolerant patients tended to express more GZMB compared to antibody-mediated rejection patients. In functional assays, IgA+ B cells exhibited high regulatory functions that were partially prevented by the presence of GZMB inhibitor. Conclusions: These data show a strong association between IgA+ and GZMB+ Bregs, particularly in tolerant kidney transplant recipients with higher GZMB and IgA expression and greater suppressive properties.
Funding Information:	KTD-INNOV (RC18_0013_01) — Dr Sophie Brouard ERA PerMed AGORA (ERAPERMED2021-086) — Dr Sophie Brouard Labex IGO (ANR-11-LABX-0016-01) — Dr Laureline Berthelot Agence Nationale de la Recherche (ANR-22-CE17-0055-01) — Dr Sophie Brouard Conseil Régional des Pays de la Loire (LOIRE-TIMES) — Dr Laureline Berthelot ABM — Dr Laureline Berthelot Société Francophone de Néphrologie, Dialyse et Transplantation — Dr Laureline Berthelot
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Date of Registration: as follow-up to "*Clinical Trials Registration*: My study was a clinical trial and is registered in one of the registries recommended by the International Committee of Medical Journal Editors (ICMJE)."	1994-01
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Key point #2: as follow-up to "Key Points: Please state the 2-3 key points of the article. The responses included here will be included with your final published paper. The key points should be complete statements and not duplications of your keywords or index terms. At least two key points are required."	IgA-expressing B cells from tolerant patients expressed granzyme B and exert regulatory functions through granzyme B production.
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If yes, please provide a list of study group(s) and members that have contributed to or participated in the submitted work in some way. as follow-up to "Study Group:	Members of the DIVAT consortium: Gilles Blancho, Julien Branchereau, Diego Cantarovich, Agnès Chapelet, Jacques Dantal, Clément Deltombe, Lucile Figueres, Raphael Gaisne, Claire Garandeau, Magali Giral, Caroline Gourraud-Vercel, Maryvonne Hourmant, Georges Karam, Clarisse Kerleau, Delphine Kervella, Christophe Masset, Aurélie Meurette, Simon Ville, Christine Kandell, Anne Moreau, Karine Renaudin, Florent Delbos, Alexandre Walencik, Anne Devis, Amory Dujardin, Angelo Testa, Gaëlle Lefrançois, Jean-François Subra, Johnny Sayegh, Julien Zuber, Christophe Legendre, Marion Rabant, Dany Anglicheau, Aude Pomerat, François Basse.

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Clinical Trial Registration My study was a clinical trial and is registered in one of the registries recommended by the International Committee of Medical Journal Editors (ICMJE).	N/A because this is not a clinical trial
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Declaration of Helsinki For all clinical experimentation described in the manuscript, I adhered to the Declaration of Helsinki and indicated my response below accordingly.	This study includes clinical experimentation and complies with the Declaration of Helsinki.
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Declaration of Istanbul as outlined in the Declaration of Istanbul on Organ Trafficking and Transplant Tourism.	
Animal Experimentation Animal experimentation is discussed in this manuscript, and I have adhered to the NIH Guide for the Care and Use of Laboratory Animals or the equivalent.	N/A
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OPEN

Kidney360 Publish Ahead of Print
DOI: 10.34067/KID.0000001054

IgA-Producing B Cells Exert Regulatory Function Through Granzyme B in Kidney Allograft Tolerance

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Abstract

Background: Tolerant kidney transplant recipients without immunosuppression have a high frequency of circulating granzyme B (GZMB)-expressing regulatory B cells (Breg). Since the precursors of these Bregs remain unknown and IgA-secreting B cells have been shown to have regulatory properties in different situations, we investigated the association between IgA- and GZMB-expressing B cells in a case-control study.

Methods: 45 healthy volunteers and 31 kidney transplant recipients with either stable graft function under immunosuppression (n=10), antibody-mediated rejection (n=7) or tolerant patients (n=14) were included. Serum immunoglobulin concentrations as glycosylation were measured by ELISA, forms of IgA were examined by Western blot. Regulatory B cells were analyzed by single-cell RNA sequencing and multiparameter spectral flow cytometry. Their function was assessed in co-cultured with T cells.

Results: Serum IgA concentration was elevated in tolerant patients compared to other transplanted patients, especially the non inflammatory IgA1 subclass, without changes in IgA size or glycosylation. Single cell transcriptomic analysis revealed higher IgA gene expression in GZMB expressing B cells and conversely higher GZMB gene expression in IgA expressing B cells. Phenotyping analysis by spectral multiparameter flow cytometry showed that IgA+ B cells were memory B cells and that IgA+ B cells from tolerant patients tended to express more GZMB compared to antibody-mediated rejection patients. In functional assays, IgA+ B cells exhibited high regulatory functions that were partially prevented by the presence of GZMB inhibitor.

Conclusions: These data show a strong association between IgA+ and GZMB+ Bregs, particularly in tolerant kidney transplant recipients with higher GZMB and IgA expression and greater suppressive properties.

Supplemental Digital Content - http://links.lww.com/KN9/B466

Introduction

Kidney transplantation typically requires lifelong immunosuppression to prevent rejection. However, discontinuation of these treatments may occur due to non-adherence difficulties and significant side effects such as infections and cancers[1, 2]. A rare group of recipients maintains stable graft function after cessation of therapy, these individuals are defined as “tolerant patients” [3, 4]. This state is associated with an enrichment of regulatory B cells (Bregs) in their blood compared to patients with stable renal function on immunosuppression and patients with rejection[5-7]. While Breg from tolerant patients have a predominant naive phenotype[5], they are able to differentiate into plasma cells and secrete more IL-10[8, 9]. Nevertheless, they do not exert their regulatory functions via IL-10 but via GZMB[10].

Other studies have found different Breg markers depending on the context, including CTLA4, TGFβ, PD-L1, IL-35, FasL, TIM-1 or IgA[11]. As our team has previously reported that B cells from tolerant patients contain a proportion of B cells expressing IgA on their cell surface membrane[12], we are further investigating the relationship between IgA and GZMB+ B cells in these patients compared to transplanted patients with stable function under immunosuppression (STA), patients with antibody-mediated rejection (ABMR) and healthy volunteers (HV), using transcriptomic and phenotypic analyses in blood. Indeed, IgA-secreting B cells have regulatory functions that are mainly based on their ability to secrete IL-10[13-15]. They are also localized at mucosal tissues in response to microbiota, but can migrate to inflamed tissues to downregulate local damage[14].

Here, in this retrospective case control study, we found that tolerant patients had a higher concentration of serum IgA than other transplanted patients, especially the IgA1 subclass, without changes in size or glycosylation of IgA. In single-cell RNA sequencing analysis, IgA expression was increased in GZMB-expressing Breg and inversely IgA-expressing B cells expressed more GZMB than non IgA-expressing B cells. Phenotyping analysis by multiparameter flow cytometry showed that IgA+ B cells were memory B cells and IgA+ B cells from tolerant patients tended to express more GZMB. Finally, in functional assays, IgA+ B cells exhibited high regulatory functions that were blocked by the GZMB inhibitor.

Methods

Patients and blood samples

Patient samples were prospectively collected in the DIVAT biocollection from a multicentric prospective French cohort of kidney and/or pancreatic transplant recipients (www.divat.fr,N° CNIL 914184, ClinicalTrials.gov recording: NCT02900040) and stored at the Biological Resource Center of the Nantes University Hospital, France (BRIF: BB-0033–00040). All patients and healthy volunteers (HV) enrolled in the study signed informed consent according to declaration of Helsinki. The clinical and research activities being reported are consistent with the Principles of the Declaration of Istanbul as outlined in the 'Declaration of Istanbul on Organ Trafficking and Transplant Tourism'. The study groups were defined as follow: (1) spontaneously tolerant patients (TOL) with stable kidney graft function (creatininemia <1.7mg/dL (<150 µmol/L) and proteinuria <1g/24h) in the absence of immunosuppression for at least 1 year (n=16), (2) stable patients (STA) with stable kidney graft function (creatininemia <1.7mg/dL (<150 µmol/L) and proteinuria <1g/24h) for at least 3 years under standard immunosuppression (calcineurin inhibitors, anti-metabolite +/- corticosteroids). (3) patients with antibody-mediated rejection (ABMR) (4) healthy volunteers (HV) without any medical history of immunosuppressive (IS) drugs or antibiotic intake in the last 6 months and also without autoimmune, inflammatory, or kidney diseases were included. All the clinical data were retrospectively extracted from the DIVAT database for kidney transplanted patients (https://integralis.chu-nantes.fr/Default.aspx) and are presented in **Table 1**. At tolerance establishment timepoint, frozen sera and cells were studied. A total of 14 TOL patients were selected among 1520 grafted patients with available serum samples and clinically defined as TOL. STA and ABMR samples were taken at the same post-transplantation timepoint to match samples. Creatininemia and proteinuria were evaluated at the same timepoint than sera and PBMC analysis. All samples were evaluated in blind experiments. The clinical status was revealed only for statistical analysis.

Serum immunoglobulin dosages

Serum concentrations of IgA, IgG, IgM, and IgA subclasses (IgA1, IgA2) were measured by ELISA. Briefly, plates (NUNC maxisorp) were coated with goat purified anti-human IgA, IgG or IgM (Bethyl Laboratories) at 1:500 in phosphate buffer saline (PBS) for 2 hours at 37°C. After incubation over night at 4°C with sera or standard serum diluted in PBS, 1% bovine serum albumin (BSA, Merck), the detection was performed using anti-Ig antibodies coupled with horseradish peroxidase (HRP) for 1 hour at 37°C. The substrate TMB (3,3',5,5'-Tetramethylbenzidine, BD biosciences) was added and the reaction stopped with sulfuric acid 0.18M. Plates were read at 450nm on Spark 10M plate reader (Tecan, Austria). For IgA1 and IgA2 quantification, plates were coated with anti-IgA1 or anti-IgA2 (Southern Biotech). Immunoglobulins were detected with mouse anti-human IgA coupled with HRP (Bethyl Laboratories) at 1:2000 dilution for 1 hour.

Measurement of IgA sialylation by ELISA

Plates (NUNC maxisorp) were coated with goat purified anti-human IgA (Bethyl Laboratories) at 1/500 in PBS for 2 hours at 37°C. After incubation over night at 4°C with sera diluted in PBS, 1% BSA. The detection was performed using the lectin SNA coupled with biotin (Sigma Aldrich) for 2 hours at 37°C. HRP coupled with streptavidin at 1/10000 was then added for 30 min The substrate TMB (BD biosciences) was added and the reaction stopped with sulfuric acid at 0.18M. Plates were read at 450nm on Spark 10M plate reader (Tecan, Austria).

Immunoblotting to detect IgA forms and size

Serum was solubilized in the SDS sample buffer under nonreducing conditions and loaded in Bolt 4-12% Bis Tris plus gels (Thermo Fischer Scientific). Proteins were electroblotted on nitrocellulose membranes (EMD Millipore, Billerica, MA) and subjected to Western blot analysis using a biotinylated goat anti-human IgA (Southern Biotechnologies) followed by donkey anti-goat IgG coupled with HRP (Jackson Immuno antibodies, Cambridge, UK). Membranes were developed by enhanced chemical luminescence treatment (GE Healthcare, Waukesha, WI) and processed with a Chemidoc machine (Biorad).

Single-cell RNA sequencing of resting B cells and Bregs

Data of single-cell RNA sequencing were obtained from the study of Sailliet N et al[9]. Briefly, B cells were purified from PBMCs by negative selection using Human B Cell Isolation Kit II and autoMACS PRO Separator, with purity >95% (Miltenyi Biotech, Gladbach, Germany). Breg stimulation was performed using CD40L (50 ng/mL) (Peprotech), rhIL-21 (10 ng/ml), F(ab')2 anti-human IgA+IgG+IgM (5 ug/ml) (Jackson ImmunoResearch), IL-2 (50 UI/ml) and CpG ODN (1 µg/ml) (Invivogen). As control, B cells were cultured in resting conditions for 48 hours. Cells were labeled with Fixable Viability Dye eFluor 450 (Invitrogen) and living cells were sorted on an ARIA II (BD Biosciences). The cells were bar-coded with anti-β2-microglobulin or anti-CD293 mAbs conjugated with DNA sequences (HashTag Oligonucleotide, HTO) following CITE-seq protocols and equivalent cell numbers were pooled. 20 000 cells were sequenced on the Nova-Seq 6000 (Illumina) at the GenoBird platform (SFR Bonamy, Nantes). Expression of *IGHA1/IGHA2* was analyzed within the clusters of GZMB Bregs and resting B cells and targeted transcript analysis was performed according to the expression of *IGHA1+IGHA2* by B cell subsets. Analysis was performed using R, Rstudio and Seurat functions.

Spectral Flow cytometry

Frozen PBMCs were thawed using serum-free thawing solution according to the manufacturer's instructions (Cellular Technology Limited) and incubated in complete RPMI 1640 medium (Invitrogen) at 37°C 5% CO2 for 1 hour. Two million of PBMCs were then stained with Live/Dead Blue viability fixable dye followed by the staining for extracellular markers performed in staining buffer (PBS with 5% m/v bovine serum albumin (BSA) and 2 mM EDTA). Intracellular staining for GZMB and IL-10 was performed using the Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences) according to the manufacture's recommendations. The antibodies used for flow cytometry analyses are listed in the **supplementary table 1**. Cells were analyzed using a Cytek AURORA spectral flow cytometer (configuration: 5L 16UV-16V-14B-10YG-8R). After individual titration of each antibody, a deconvolution matrix was set up according to Cytek recommendations and quality control procedure was performed prior each data acquisition. Data treatment and analyses were performed using OMIQ software (Dotmatics, www.dotmatics.com). Each file was first cleaned using the PeacoQC algorithm and, after the removal of debris, doublets, dead cells, and auto-fluorescent cells, B cells were identified as CD3- CD14- CD16- CD56- HLA-DR+ CD20+ CD19+ and subjected to supervised analysis.

B and T cell coculture to evaluate regulatory function

B cells were purified from PBMCs of healthy blood donors by negative selection using Human B Cell Isolation Kit II and autoMACS PRO Separator, with purity >95% (Miltenyi Biotech, Gladbach, Germany), thereafter CD3- CD14- IgA+/- B cells were sorted on an ARIA III flow cytometry cell sorter (BD Biosciences, Le Pont-de-Claix, France). $IgA^{+/-}$ B cells (1 million/mL) were cultured for 24h at 37°C 5% CO2 in complete RPMI-1640 medium containing L-glutamine, penicillin/streptomycin (Life Technologies, Carlsbad, CA), and 10% FCS (Lonza, Verviers, Belgium) and activated or not during 24h with a combination of CD40L (50 ng/mL, Peprotech), recombinant human IL-21 (10 ng/mL, Miltenyi Biotech), F(ab')2 anti-human IgA+IgG+IgM (5 µg/ml, Jackson ImmunoResearch), IL-2 (50 UI/mL) and CpG ODN (1 µg/mL, Invivogen). The next day, CD4+CD25- naïve T cells were purified among resting PBMC by negative selection using CD4+ T Cell Isolation Kit II (Miltenyi Biotech), according to the manufacturer's instructions with a purity >95%. $CD4^{+}CD25^{-}$ T cells were stained with Cell Proliferation Dye eFluor 450 (CPD) (Thermo Fisher Scientific) to evaluate proliferation. CPD-labeled $CD4^{+}CD25^{-}$ T cells were activated with CD3/CD28 Dynabeads (Invitrogen) at a T cell/bead ratio of 1:1 in 96-well, U-bottom plate at 37°C with 5% CO2. The aforementioned B cells were added to the plate with activated $CD4^{+}CD25^{-}$ T cells at a ratio of B:T cells 2:1 for 3 days. At the end of the coculture, viability staining was performed using the aqua Live/dead cell staining kit (Thermo Fisher Scientific) and the proliferation of $CD4^{+}CD25^{-}$ T cells was assessed by flow cytometry using Canto II flow cytometer (BD Biosciences). GZMB inhibitor (GZMB inhibitor IV, Calbiochem Research Biochemicals) or anti-IL-10 blocking antibody (clone JES3-19F1, BD Biosciences) were used at 10 µg/mL.

Statistical Analysis

All data were expressed as the median ± interquartile range (IQR). Data from the different groups were compared using the Kruskal Wallis test with Dunn's post hoc tests for multigroup comparisons. Gene differential expression p values were calculated using packages of Seurat and MAST v1.16.0, which uses a model tailored for the zero-enriched scRNAseq datasets. P-values were automatically adjusted with the test number by the function FindMarkers of Seurat. Adjusted p-values are shown and differences were defined as statistically significant when $p<0.05$. Analyses and graphical representation were performed using GraphPad Prism v.10 (GraphPad Software, La Jolla, CA, USA).

Results

IgA concentrations are increased in sera from TOL patients compared to STA and ABMR patients without modification of IgA forms and sialylation levels.

To investigate humoral changes associated with tolerance, we first examined immunoglobulin concentrations in sera from healthy volunteers (HV), TOL, STA and ABMR patients (**Table 1**). TOL patients exhibited a decrease of IgM concentrations (1.16 [0.75-1.69] g/L, $p<0.05$) (**Figure 1a**) compared to STA (2.04 [1.19-3.1] g/L) and ABMR patients (1.91 [1.157-2.96] g/L), while IgG concentrations were similar across all groups (**Figure 1a**). TOL patients also exhibited increased IgA concentrations (median=2.3 [1.67-2.81] g/L, $p=0.0291$) with equivalent levels to HV (2.51 [1.93-3.08] g/L), compared to other transplanted patients (STA 1.51 [1.38-2.76], ABMR 1.62 [1.30-2.19] g/L) (**Figure 1b**). Analysis of IgA1 and IgA2 subclasses revealed an increase of IgA1 concentrations in TOL patient serum (1.91 [1.58-2.75] versus STA 1.4 [1.33-1.78], ABMR 1.55 [1.25-2.02] g/L, $p<0.05$) and no differences for IgA2 (HV 0.097 [0.06-0.17], TOL 0.067 [0.038-0.098], STA 0.077 [0.05-0.12], ABMR 0.066 [0.029-0.10 g/L] (**Figure 1b**). Given the known immunomodulatory differences between monomeric and polymeric IgA[16], we assessed the molecular forms of serum IgA using immunoblotting. Both monomeric (~160 kDa) and dimeric (~350 kDa) IgA were present, but their relative proportions did not differ significantly between groups (**Figure 1c**). Moreover, IgA sialylation levels, an indicator of glycan-mediated immunoregulatory function, were unchanged (**Figure 1d**) suggesting a quantitative rather than qualitative alteration in IgA in tolerant patients.

IgA expression is enriched in GZMB+ Bregs from TOL patients

As IgA concentrations and GZMB+ Bregs are both increased in the blood of TOL patients, we further examined the relationship between IgA and GZMB Bregs. Using single-cell transcriptomic analysis, we evaluate the expression of *IGHA1* and *IGHA2* mRNA in *in vitro* expanded GZMB+ Bregs and resting B cells from HV, TOL, STA and ABMR patients according to previously published protocol[12]. As previously shown, the frequency of GZMB+ B cells among total B cells was increased in TOL patients compared to all groups (Suppl Table 2). The UMAP analysis showed two clusters of cells separating resting B cells (left cluster) from GZMB+ Bregs (right cluster) (**Figure 2a**). IgA-expressing B cells (*IGHA1*+ and *IGHA2*+) were found in both resting B cells and Bregs. Interestingly, TOL patients exhibited a higher

frequency of IgA+ cells within the GZMB+ Breg cluster as compared to the other groups of patients (54% of *IGHA1* and 14% of *IGHA2* among GZMB+ Bregs in TOL patients as compared to 15-29% of *IGHA1* and 1-9% of *IGHA2* for other STA and ABMR; **Figure 2b**). The level mean of IgA expression per cell were not different between the different groups of patients (*data not shown*). IgA-expressing cells in GZMB+ Bregs and in resting B cells expressed markers of differentiated cells (*CD27, XBP1* and *IRF4*) (**Figure 2c**). In the same way, *IGHA1*/*IGHA2* expressing B cells significantly expressed more *GZMB* mRNA than IgA non expressing B cells (**Suppl Table 3**).

Using multiparameter spectral flow cytometry, we analyzed the phenotype of IgA+ B cells in blood from TOL patients compared with other transplanted patients (STA, ABMR) and HV (**Figure 3a**). TOL patients exhibited no differences of IgA+ B cell frequencies (7.78 [3.93-11.63] %) compared to HV (10.15 [7.81-13.63] %), and STA patients (7.52 [3.33-12.73] %, **Figure 3b**). However, the frequencies of IgA+ B cells were significantly decreased in ABMR patients (4.16 [1.32-5.37] %) compared to HV ($p=0.042$, **Figure 3b**). In accordance with single cell data, IgA+ B cells exhibited a memory B cell phenotype in all groups of patients with two predominant $CD27^{+}IgD^{-}$ (switch memory) and $CD24^{high}CD38^{low}$ (memory) phenotypes (**Figure 3c and 3d**). We then assessed the expression of GZMB in IgA+ B cells (**Figure 3e**). Interestingly, IgA+ B cells from TOL patients expressed more GZMB (2.75 [2.01-5.92] %) than HV (0.97 [0.60-2.01] %) and ABMR patients (2.00 [0.41-3.31] %). CD25 (or IL2RA, the α chain of IL-2 receptor) is a marker of activation. The frequency of CD25+ IgA+ B cells was increased in all patient groups (TOL 7.61 [4.33-18.05], STA 11.85 [8.06-13.68], ABMR 8.52 [8.38-12.68] %) compared to HV (4.36 [3.56-7.31] %). To note, STA patients exhibited an increase of PD1+ IgA+ B cell frequency (1.55 [0.39-2.47], $p<0.05$) compared to all groups (0.64 [0.17-1.04] % in HV, 0.09 [0.00-0.58] % in TOL and 0.43 [0.10-0.81] % in ABMR). CD11c expression was not different in all groups. Collectively, these data suggest that TOL patients exhibited an accumulation of IgA+ memory B cells expressing GZMB in their blood.

GZMB+ Breg progenitors are enriched in IgA+ B cells whose suppressive properties depend on GZMB and not on IL-10.

GZMB+ Bregs and IgA+ B cells have been shown to exhibit regulatory activities through the inhibition of various cell types, particularly through the inhibition of CD4 and CD8+ T cell proliferation. Using a protocol previously established by our team for in vitro GZMB+ Breg expansion[12], we investigated whether IgA+ B cells could exert the same inhibitory functions. While all B cell subsets were able to inhibit the proliferation of CD4+ T cells, IgA+ B cells were the most suppressive population (34.3% [16.0-56.2] vs 56.0% [43.4-78.1] of CPD^{low} CD4+ T cells co-cultured with IgA+ and IgA- B cells respectively; p=0.0267, **Figure 4a**). GZMB inhibitor was able to partially prevent the suppressive function of total B cells as IgA+ B cells (**Figure 4b**). As expected, unstimulated B cells were not able to suppress the proliferation of activated CD4+ T cell (**Figure 4c**). As for total B cells, the suppressive function of IgA+ B cells was not dependent on IL-10, as the blockade of IL-10 has no effect on the proliferation of CD4+ T cells (**Figure 4d**). Collectively, we demonstrated that the GZMB+ Bregs are enriched within IgA+ B cells, whose suppressive properties depend partially on GZMB.

Discussion

Kidney allograft tolerance without immunosuppression is associated with circulating Bregs acting through the GZMB protease[10]. As such cells are rare; we are developing a method to expand them *in vitro* while maintaining their suppressive properties[12], paving the way for a possible cell therapy. We report here that TOL patients have higher concentration of circulating IgA, specifically the IgA1 subclass, and that TOL patients also have more circulating IgA+ B cells with a memory phenotype and expressing GZMB. Finally, these IgA+ B cells exhibited regulatory functions acting through GZMB. Thus, TOL patients have more IgA+ Bregs expressing GZMB and exhibiting inhibitory function.

Bregs have historically been defined as IL-10-secreting B cells, but it is now well established that Bregs are a pool of distinct B cells, each being characterized by different markers (IL-35, TGF-β, GZMB, IDO, PD-L1, CD1d, CD25, CD39/CD73, TIM-1, IgA), all of which share regulatory functions[17]. In transplantation, while we report an increased number of GZMB-expressing B cells in the blood of TOL patients acting through GZMB and not IL-10[10], TIM1+ IL-10+ Bregs have been associated

with transplant tolerance in mouse models [18]. Conversely, kidney transplant patients with ABMR have Bregs with an altered IL-10/TNFα pathway[19]. So, depending on the immune context, the markers of Breg differ, but B cell-mediated immune regulation appears as a key element for kidney transplant tolerance. We report here that IgA is a potential marker associated to GZMB in renal allograft tolerance. As GZMB is an intracellular marker, the expression of IgA at cell surface on those cells is of interest for monitoring, cell sorting and potentially cell therapy.

The IgA class is the second Ig in the blood after IgG and the most produced Ig class by the body, IgA being constantly secreted at the mucosal surface[20]. IgA is predominantly monomeric in the blood, whereas in secretion it is dimeric, linked by a J chain, which carries the secretory component and is called secretory IgA (SIgA). Monomeric IgA induces anti-inflammatory responses in myeloid cells bearing the Fc IgA receptor (FcαRI, CD89) and, on the other hand, polymeric IgA binding to FcαRI results in cell activation and inflammation.[21] There are also two IgA subclasses: IgA1 and IgA2, which differ in their hinge region, the hinge region of IgA1 being longer and containing O-glycosylation sites. Defects in IgA1 glycosylation can lead to IgA deposition in the mesangium, as observed in patients with IgA nephropathy[22] and recurrence of IgA deposition after kidney transplantation[23]. In addition, sialylation of IgG was affected in kidney transplant patients[24, 25]. Here, we showed an increase of blood IgA in TOL patients with no modification of IgA forms, the majority of IgA being monomeric IgA1. All transplanted patients showed hyposialylation of IgA, as previously observed for IgG[24]. There was no difference of IgG concentrations in all groups, indicating that immunosuppression did not probably induce the specific decrease of IgA in STA and ABMR patients. IgA deficiency (IgA concentration <0,07 mg/dL) is frequent in the general population (1/600 in France)[26] and IgA concentrations vary greatly from one individual to another (0.8 to 4 g/L). IgA deficiency is associated to an increase of inflammation and allergy. This suggest that TOL patients could be naturally high IgA producers, favoring tolerance.

Intriguingly, PD-1, an immune checkpoint molecule able to induce an inhibitory signal when it binds one of its ligands (PD-L1 and PD-L2)[27], was upregulated in IgA+ B cells from STA patients compared with HV, TOL and ABMR patients. PD-1 is also described as an exhaustion marker on virus-specific B cells[28]. So, IgA+ B cells from STA patients could be easily inhibited whereas B cells from other groups could not. In another hand, PD-1 deficiency impairs B cell memory and antibody responses[29],

suggesting a more complex role of PD-1 on B cells. Another hypothesis to explain the PD-1 expression increase in STA patients versus TOL patients could be that PD-1+ B cells are inflammatory cells as demonstrated in rheumatoid arthritis[30].

IgA+ B cells are closely associated with the microbiota[20, 31], with their main localization in mucosal tissues, where they secrete dimeric IgA, which are excreted in luminal fluids and participate in the immune exclusion of pathogens and the regulation of microbiota composition[20]. Chronic kidney diseases are associated with gut microbiota dysbiosis, and microbiota intervention can prevent kidney injury in several models[32]. Compared to healthy individuals, the intestinal and urinary microbiota are also altered in transplant patients with stable graft function[33-37] and more drastically in patients with ABMR[37-39]. Furthermore, the close relationship between immunosuppressants and gut microbiota has implications for the health of kidney transplant recipients[38]. However, little is known about microbiota in transplant tolerance, excepted for the high diversity of bacteria with a Proteobacteria profile in the urinary microbiota of TOL patients[40]. Interestingly, reciprocal interactions between Breg and microbiota are important in graft tolerance, with Breg function being modulated by microbiota diversity as shown in skin grafts[41].

Investigating IgA-microbiota interactions in the context of tolerance will be a new challenge, as will investigating the specific antigens recognized by IgA+ B cells in tolerant patients, exploring the role of IgA in the gut microbiome in transplant tolerance, and investigating the long-term stability of IgA+ B cell populations in tolerant patients. Moreover, definitive conclusions on IgA+ Breg role towards tolerance will be drawn by the use of transplantation animal models.

In conclusion, our study highlights the potential role of IgA+ B cells in post-transplant allograft tolerance. Further studies are needed on larger cohorts to replicate and validate these findings. In addition, further mechanistic studies are needed to fully understand the interplay between IgA+ GZMB+ B cells and other immune cells in transplant tolerance.

Acknowledgements

We thank the patients who participated in the study and the physicians who helped to recruit the patients. We thank the DIVAT biocollection (www.divat.fr, n°CNIL 914184, ClinicalTrials.gov identifier: NCT02900040) and the biological resource center for biobanking (CHU Nantes, Hôtel Dieu, Tumorothèque, Nantes, F-44093, France) for sample collection and storage.

Patient groups	HV (n=45)	TOL (n=14)	STA (n=10)	ABMR-1 (n=7, ELISA)	**ABMR-2 (n=8, Flow cytometry)**
Recipient age: median (IQR)	41.0 (28.1-54.3)	37.0 (30.0-55.5)	57.5 (47.7-61.7)	42.0 (40.0-61.0)	52,5 (30.1-65.2)
Recipient sex ratio: Male/Female number	23/22	9/5	7/3	5/2	5/3
Donor age: median (IQR)	NA	31.5 (17.7-39.7) [a]	55.0 (46.5-61.0)	37.0 (30.0-37.0)	53.4 (44.5-60.3)
Donor status: living/non-living donor number	NA	11/3 [b]	0/10 [b]	1/6 [b]	0/8 [b]
HLA mismatches: median (IQR)	NA	3.0 (0.2-4.0) [c]	4.0 (2.7-5.0)	2.0 (2.0-5.0)	3.6 (3.0-5.0)
Presence of DSA: ND/Positive/Negative DSA number	NA	2/2/10	0/0/10	1/1/5 [d]	0/8/0 [d]
Time post-transplantation (months): median (IQR)	NA	307.9 (262.0-402.9) [e]	136.7 (113.1-165.0)	218.0 (100.5-284.6)	224.3 (55.1-302.5)
Creatinemia (mg/dL or *µmol/L*): median (IQR)	NA	1.12 (1.03-1.66) *99.0 (79.0-128.0)*	1.50 (1.07-1.54) *116.0 (95.2-136.3)*	2.82 (1.89-3.40) [f] *250.0 (167.0-301.0)* [f]	1.90 (1.49-2.49) [f] 168.1 (131.8-220.3) [f]
Proteinuria (g/24h) : median (IQR)	NA	0.1 (0.0-0.4)	0.2 (0.1-0.4)	2.5 (1.6-6.2) [g]	2.0 (1.3-5.1) [g]

Table 1 : Clinical and demographic characteristics of patients. HV= healthy volunteers, TOL= kidney operational tolerant patients, STA= stable patients, ABMR = antibody-mediated rejection patients, SD= standard deviation, DSA= donor specific antibody , NA= not applicable, ND=not determined. ABMR-1 patient group samples were assessed by ELISA, Western blot and scRNAseq. ABMR-2 samples as HV, TOL and STA with available frozen PBMCs were used in flow cytometry experiment. One-way anova with Kruskal-Wallis test for mulitple comparison were used for comparison of median and Fisher's exact test for contingency data. a: TOL vs STA p=0.0014. b: TOL vs STA TOL vs ABMR1, TOL vs ABMR2, p<0.0001. c: TOL vs STA p=0.043, TOL vs ABMR2 p=0.0032. d: ABMR1&2 vs TOL and STA, p<0.0001. e: TOL vs STA,TOL vs ABMR1 p<0.005. f: ABMR1&2 vs TOL, ABMR1&2 vs STA p=0.0005. g: ABMR1 vs TOL p=0.012, ABMR1 vs STA p=0.0004, ABMR2 vs TOL p=0.013, ABMR2 vs TOL p=0.0005.a: TOL vs STA p=0.0014. b: TOL vs STA vs ABMR, p<0.0001. c: TOL vs STA p=0.043. d: TOL vs STA p<0.0001,TOL vs ABMR p=0.0045. e: ABMR vs TOL p=0.0005, ABMR vs STA p=0.0005. f: ABMR vs TOL p=0.012, ABMR vs STA p=0.0004.

Figure Legends

Figure 1: Serum IgA concentrations are increased in TOL patients compared to STA and ABMR. **a.** Dosages of IgM and IgG concentrations in sera of patients. **b.** Dosages of IgA, IgA1 and IgA2 concentrations in sera by ELISA. **c.** One representative Immunoblot for serum IgA. Sera IgA concentrations were normalized to charge 100ng of IgA per well to compare forms of IgA and not quantity. Monomeric IgA appear around160kDa, dimeric IgA at 350kDa. Quantification of band intensity is presented in the middle graph, the ratio of dimer/monomers is shown on the left right graph. **d.** Sialylation of serum IgA was assessed by ELISA using Sambucus Nigra (SNA) lectin.
HV= healthy volunteers, TOL= tolerant, STA= stable, ABMR=antibody mediated rejection patients.

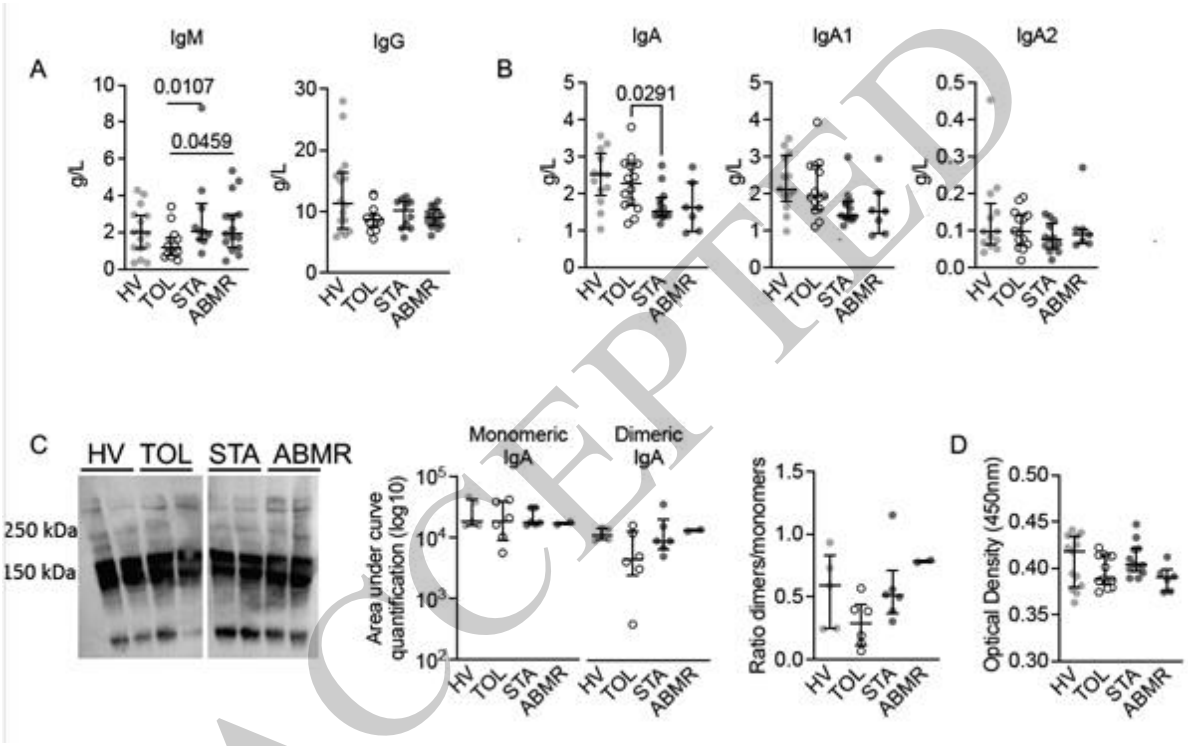


Figure 2: Single-cell RNA sequencing revealed an enrichment of IgA expressing B cells in GZMB expressing Bregs in TOL patients. Transcriptomic of differentiated *in vitro* GZMB+ B cells was compared to resting B cells at single cell level. **a.** Uniform Manifold Approximation and Projection for Dimension Reduction (UMAP) of B cells. Two clusters appeared, one corresponding to resting B cells (right population in UMAP) and the other one to GZMB+ Breg (left cluster in UMAP). *IGHA1* and *IGHA2*, the two genes coding IgA1 and IgA2 subclasses (heavy chains) expressed in IgA+ B cells, were more expressed in the GZMB+ Breg cluster. **b**. Percentages of B cells expressing *IGHA1* and *IGHA2* among resting B cells and GZMB+ Breg cells. **c**. Expression of B cell genes of interest among IgA+ and IgA- B cells. HV= Healthy Volunteers,TOL= tolerant, STA= stable, ABMR=antibody mediated rejection patients.

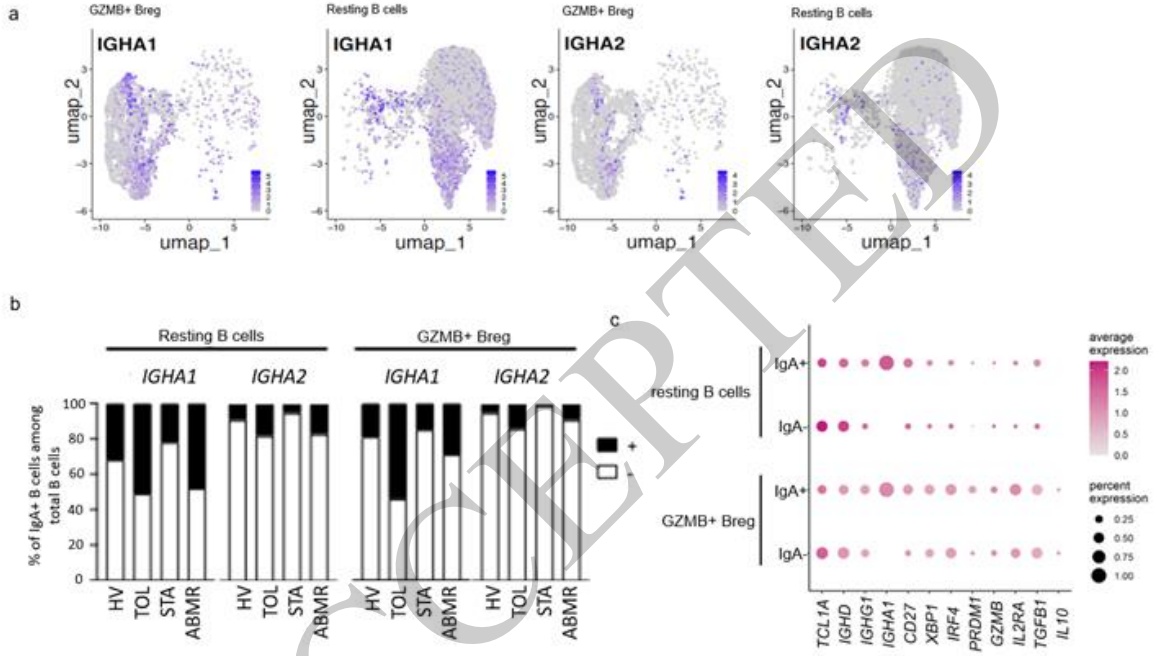


Figure 3: Characterization of blood IgA+ B cells by spectral flow cytometry. a. Graphical summary of patient groups and studied markers. **b.** Frequencies of IgA+ B cells among patient groups. **c** Relative frequencies of B cell subsets among patient groups using CD27 and IgD markers. **d.** Relative frequencies of B cell subsets among patient groups using CD38 and CD24 markers. **e.** Expression of GZMB, CD25, PD1 and CD11c markers among IgA+ B cells. HV= healthy volunteers, TOL= tolerant, STA= stable, ABMR=antibody mediated rejection patients.

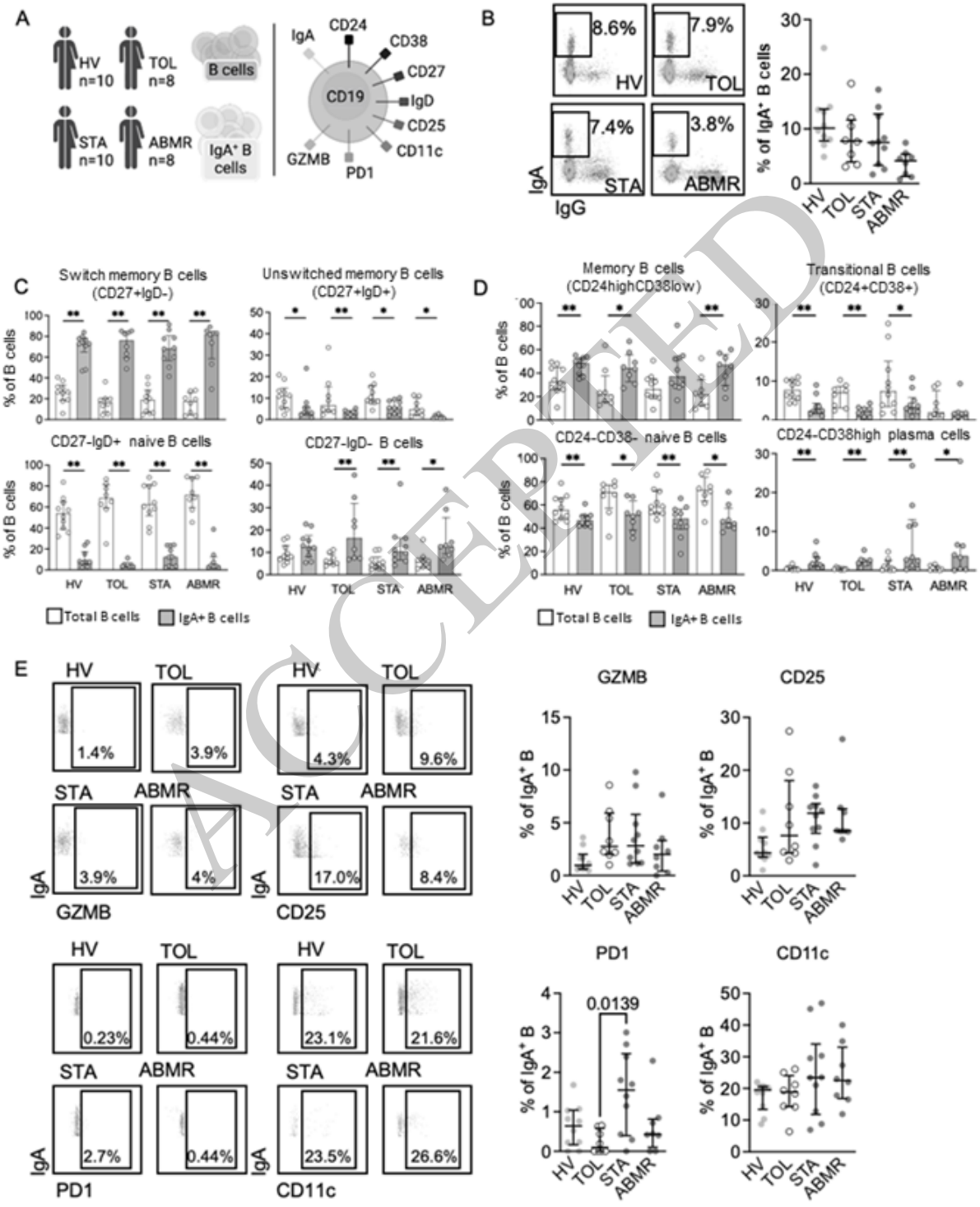
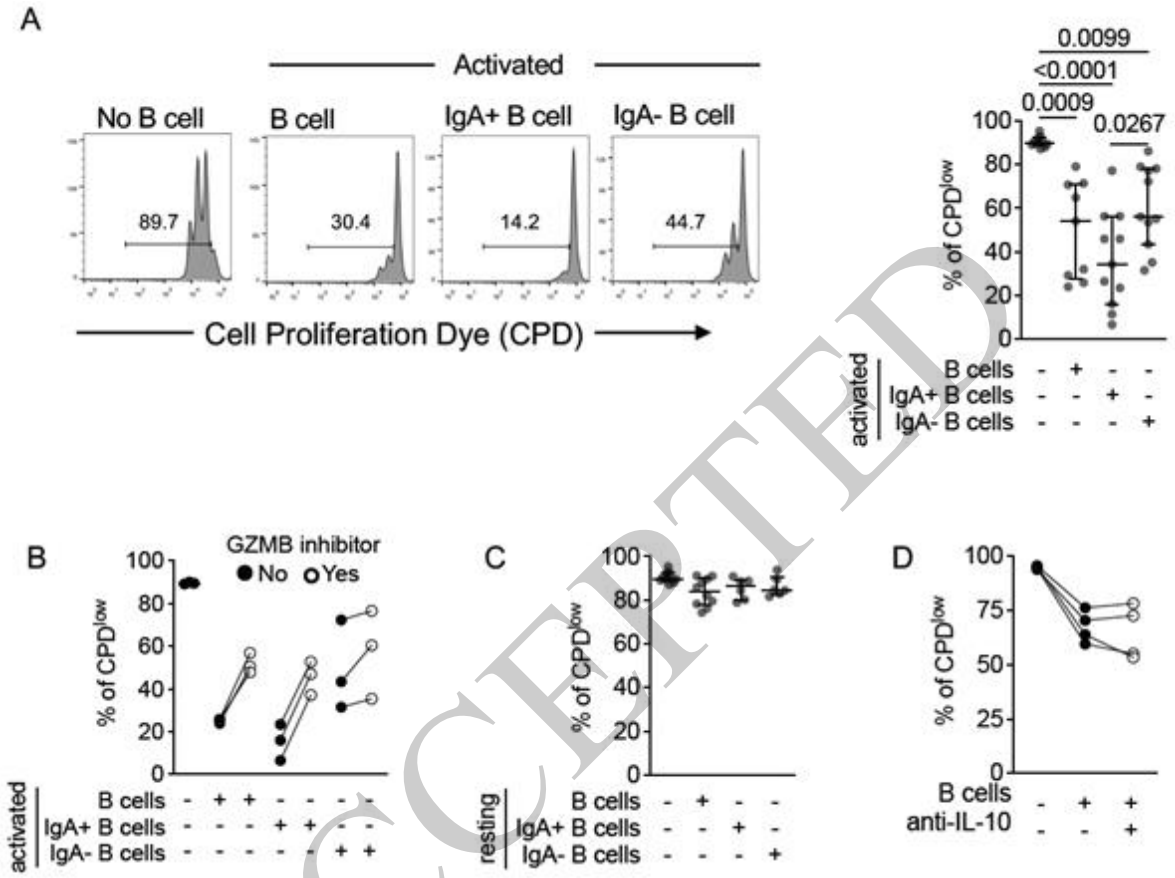


Figure 4: IgA+ B cells exert higher inhibitory function than IgA- B cells through GZMB. a. T cell proliferation in co-culture with activated B cells, representative dot plots for each condition and total quantification (left graph). **b.** T cell proliferation in co-culture with activated B cells and GZMB inhibitor. **c**. T cell proliferation in co-culture with resting B cells. **d**. T cell proliferation in co-culture with activated B cells and anti-IL-10 blocking antibody.



Supplemental Table of Contents

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L. Berthelot reports the following:
Employer: INSERM UMR 1064

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Laureline Berthelot
Manuscript ID: K360-2025-000113R2
Manuscript Title: IgA-production B cells exert regulatory function through granzyme B in kidney allograft tolerance
Date of Completion: October 14, 2025
Disclosure Updated Date: October 14, 2025

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L. Boussamet reports the following:
Employer: INSERM UMR 1064

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Léo Boussamet
Manuscript ID: K360-2025-000113R2
Manuscript Title: IgA-production B cells exert regulatory function through granzyme B in kidney allograft tolerance
Date of Completion: October 14, 2025
Disclosure Updated Date: October 14, 2025

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F.Brinas reports the following:
Employer: INSERM UMR 1064, IUCPQ U.Laval

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: François Brinas

Manuscript ID: K360-2025-000113R2

Manuscript Title: IgA-production B cells exert regulatory function through granzyme B in kidney allograft tolerance

Date of Completion: October 14, 2025
Disclosure Updated Date: October 17, 2025

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S. Brouard has nothing to disclose.

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Sophie Brouard
Manuscript ID: K360-2025-000113R2
Manuscript Title: gA-production B cells exert regulatory function through granzyme B in kidney allograft tolerance
Date of Completion: October 17, 2025
Disclosure Updated Date: March 9, 2025

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L. Cappelier reports the following:
Employer: Inserm UMR1064

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Laura Cappelier
Manuscript ID: K360-2025-000113R2
Manuscript Title: IgA-production B cells exert regulatory function through granzyme B in kidney allograft tolerance
Date of Completion: October 14, 2025
Disclosure Updated Date: October 14, 2025

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M. Chesneau has nothing to disclose.

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Mélanie Chesneau
Manuscript ID: K360-2025-000113R2
Manuscript Title: IgA-producing B cells exert regulatory function through granzyme B in kidney allograft tolerance.
Date of Completion: October 28, 2025
Disclosure Updated Date: October 28, 2025

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L. Colas reports the following:
Consultancy: Abbvie, ALK, Astra-Zénéca, GSK Pharma, Novartis, Sanofi-Aventis, Stallergène-Greer, Thermo-Fisher.

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Luc Colas
Manuscript ID: K360-2025-000113R2
Manuscript Title: IgA-production B cells exert regulatory function through granzyme B in kidney allograft tolerance
Date of Completion: October 17, 2025
Disclosure Updated Date: October 14, 2025

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R. Danger reports the following:
Employer: INSERM

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Richard Danger
Manuscript ID: K360-2025-000113R2
Manuscript Title: IgA-production B cells exert regulatory function through granzyme B in kidney allograft tolerance
Date of Completion: October 14, 2025
Disclosure Updated Date: October 14, 2025

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N. Degauque reports the following:
Employer: Nantes Université, INSERM, Center for Research in Transplantation and Translational Immunology (CR2TI), UMR 1064

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Nicolas Degauque
Manuscript ID: K360-2025-000113R2
Manuscript Title: IgA-production B cells exert regulatory function through granzyme B in kidney allograft tolerance
Date of Completion: October 15, 2025
Disclosure Updated Date: October 15, 2025

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A. Dupuy has nothing to disclose.

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Amandine Dupuy
Manuscript ID: K360-2025-000113R2
Manuscript Title: IgA-production B cells exert regulatory function through granzyme B in kidney allograft tolerance
Date of Completion: October 14, 2025
Disclosure Updated Date: October 14, 2025

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M. Giral has nothing to disclose.

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Magali Giral
Manuscript ID: K360-2025-000113R2
Manuscript Title: IgA-producing B cells exert regulatory function through granzyme B in kidney allograft tolerance
Date of Completion: October 31, 2025
Disclosure Updated Date: October 17, 2025

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C. Kerleau reports the following:
Employer: CHU Nantes

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Clarisse Kerleau
Manuscript ID: K360-2025-000113R2
Manuscript Title: IgA-production B cells exert regulatory function through granzyme B in kidney allograft tolerance
Date of Completion: October 14, 2025
Disclosure Updated Date: October 14, 2025

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H. Mai has nothing to disclose.

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Hoa Le Mai
Manuscript ID: K360-2025-000113R2
Manuscript Title: IgA-production B cells exert regulatory function through granzyme B in kidney allograft tolerance
Date of Completion: October 16, 2025
Disclosure Updated Date: October 16, 2025

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C. Mathé reports the following:
Employer: Chelatec

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Camille Mathé
Manuscript ID: K360-2025-000113R2
Manuscript Title: IgA-production B cells exert regulatory function through granzyme B in kidney allograft tolerance
Date of Completion: October 14, 2025
Disclosure Updated Date: October 14, 2025

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N. Sailliet has nothing to disclose.

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Nicolas Sailliet
Manuscript ID: K360-2025-000113R1
Manuscript Title: IgA-producing B cells exert regulatory function through granzyme B in kidney allograft tolerance.
Date of Completion: September 29, 2025
Disclosure Updated Date: September 29, 2025

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G. Tilly reports the following:
Employer: nantes universite

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Gaelle Tilly
Manuscript ID: K360-2025-000113R2
Manuscript Title: IgA-production B cells exert regulatory function through granzyme B in kidney allograft tolerance
Date of Completion: October 15, 2025
Disclosure Updated Date: October 15, 2025

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N. Yeremenko reports the following:
Employer: Nantes University

I understand that the information above will be published within the journal article, if accepted, and that failure to comply and/or to accurately and completely report the potential financial conflicts of interest could lead to the following: 1) Prior to publication, article rejection, or 2) Post-publication, sanctions ranging from, but not limited to, issuing a correction, reporting the inaccurate information to the authors' institution, banning authors from submitting work to ASN journals for varying lengths of time, and/or retraction of the published work.

Name: Nataliya Yeremenko
Manuscript ID: K360-2025-000113R2
Manuscript Title: IgA-producing B cells exert regulatory function through granzyme B in kidney allograft tolerance,
Date of Completion: November 18, 2025
Disclosure Updated Date: November 18, 2025